THIS PAGE BLANK (USPTO)

Europäisches Patentamt

European Patent Office

Office européen des brevets

⑪ Publication number: **0 113 328**
A2

⑫ **EUROPEAN PATENT APPLICATION**

㉛ Applicant: **Monsanto Company**
Patent Department 800 North Lindbergh Boulevard
St. Louis Missouri 63167(US)

㉚ Inventor: **Feder, Joseph**
935 Mulberry Lane
University City Missouri 63130(US)

㉚ Inventor: **Lewis, Charles, Jr.**
2075 Gerard Park Lane
Hazelwood Missouri 63042(US)

㉚ Inventor: **Tolbert, William Robert**
1415 Montdore Drive
Manchester Missouri 63011(US)

㉔ Representative: **Lunt, John Cooper et al,**
Monsanto Europe S.A. Patent Department Avenue de
Tervuren 270-272 Letter Box No 1
B-1150 Brussels(BE)

㊄ Static cell culture maintenance system.

㊗ In apparatus for maintaining animal cells in vitro in a substantially arrested state of proliferation with continuous secretion of cell product, the cells are retained within a reactor vessel chamber (39) in a semi-rigid matrix having interstices for passage of fluid nutrient medium. Fresh nutrient medium is supplied by perfusion into the matrix through relatively low porosity tubes (40) which are suspended in the reactor chamber (39) and which substantially traverse the matrix; expended medium and cell product is withdrawn through relatively high porosity tubes (41) which are also suspended in the reactor chamber (39) and which substantially traverse the matrix; and oxygenated gaseous medium is supplied by perfusion into the matrix through a selectively-permeable membrane (42) disposed in the reactor chamber (39).
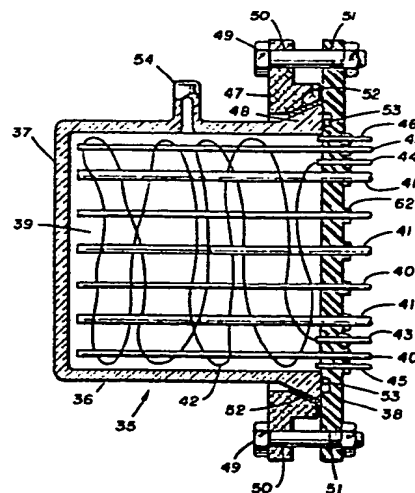
FIG. 3.

EP 0 113 328 A2

STATIC CELL CULTURE
MAINTENANCE SYSTEM

Background of the Invention

This invention relates to a method and
apparatus for cell culture and more particularly
to a static cell culture maintenance system.

5   The culture of animal cells in vitro for
the production of various proteins, peptides,
hormones, growth factors and other biologically
active substances has been widely investigated. A
great many animal cells have been utilized in cell
10   culture to secrete important biomolecules which
are of significant biomedical interest. For
example, pituitary cells have been cultured in
vitro to produce growth hormone; kidney cells have
been cultured to produce plasminogen activator;
15   and hepatitis-A antigen has been produced in
cultured liver cells. Other cells have been
specifically cultured to produce various viral
vaccines and antibodies. So also, interferon,
insulin, angiogenic factor, fibronectin and
20   numerous other biomolecules have been produced by
the in vitro culture of various animal cells.

A great diversity of procedures and
apparatus for in vitro cell culture production of
biomolecules of interest has been utilized
25   heretofore. In certain relatively simple systems,
the cells are grown to confluence in tissue flasks
and roller bottles in the presence of suitable
nutrient media. More complex systems have
utilized capillary hollow fiber membranes as the

surface support and as the means of
supplying nutrient media to the cells. In the
latter systems, nutrient culture media can be
pumped through the lumen of hollow fibers arranged
5   in an elongated bundle as described in U.S.
Patents 3,821,087; 4,220,725; and 4,184,922; or
oxygen can be supplied through the hollow fiber
membranes to maintain aerobic conditions as
disclosed in U.S. Patent 3,997,396.

10   A further improvement of the hollow fiber
membrane cell culture technology employs a flat
bed configuration in which the nutrient medium is
supplied transverse to the bed of fibers in a
relatively short flow path as seen from U.S.
15   Patents 4,087,327 and 4,201,845. The flat bed
configuration reduces the undesirable nutrient
gradient produced by the elongated cartridge or
bundle configuration of the hollow fiber
membranes.

20   Other cell culture systems propogate the
cells in agitated liquid suspension culture,
particularly for larger scale operations as
described, for example, in U.S. Patents 4,059,485;
4,166,768; 4,178,209; and 4,184,916. In the case
25   of cells which require surface support,
microcarriers have been employed in the suspension
culture medium as the support means. Such
microcarriers are illustrated, for example, in
U.S. Patents 3,717,551; 4,036,693; 4,189,534;
30   4,203,801; 4,237,033; 4,237,218; 4,266,032;
4,289,854; 4,293,654; and 4,335,215.

Further background information on conventional culture conditions for the production of animal cells in microcarrier culture can be had by reference to the recent paper by Clark and Hirtenstein, Ann. N.Y. Acad. Sci. 369, 33-45 (1981).

In most of the aforesaid in vitro cell culture systems, emphasis has been placed on means to stimulate proliferation of large numbers of cells or for investigation of differentiated function of very few non-proliferating cells. Although some secreted biomolecules can be produced during periods of high proliferation, more efficient production of such export biomolecules can be obtained in arrested, more differentiated states. Moreover, some in vivo secretory cells have very low proliferation rates. Accordingly, an in vitro cell culture system which allows a large number of cells to be maintained in a state of low proliferation but with continuous product secretion would be of significant value.

Brief Description of the Invention

In accordance with the present invention animal cells are maintained in vitro in a substantially arrested state of proliferation with continuous secretion of cell product by a system which comprises:

(a) suspending said cells in a semi-rigid matrix disposed in a cell culture reactor chamber and having interstices for passage of fluid nutrient medium,

5   (b) supplying fresh nutrient medium for said cells by passing said nutrient medium through an external inlet in the reactor into the lumen of at least one first porous tube disposed in said chamber substantially throughout the expanse of

10   said matrix and perfusing said nutrient medium through the walls of said porous tube into said matrix,

(c) withdrawing expended nutrient medium and cell product from said matrix by perfusing

15   through the walls of at least one second porous tube disposed in said chamber substantially throughout the expanse of said matrix and passing said spent medium and cell product through an external outlet in said reactor, said second

20   porous tube having a pore size larger than the pore size of said first porous tube, and

(d) supplying oxygenated gaseous medium for said cells by perfusing into said matrix through the walls of at least one selectively-permeable

25   tubular membrane disposed in said chamber substantially throughout the expanse of said matrix and having openings at both ends of its lumen for inlet and outlet of said gas to and from the outside of said reactor.

07-21-(65)A

0113328

The apparatus used in this cell maintenance system comprises a housing, a chamber within said housing for holding cells in a semi-rigid matrix with interstices for passage of fluid culture media, external culture medium inlet and outlet means disposed in a wall in said housing, said inlet means being in fluid communication with at least one relatively low porosity tube disposed substantially throughout the expanse of said chamber, said outlet means being in fluid communication with at least one relatively high porosity tube disposed substantially throughout the expanse of said chamber, external gas inlet and outlet means disposed in a wall in said housing and in gaseous communication with a selectively-permeable membrane disposed substantially throughout the expanse of said chamber, said relatively low and relatively high porosity tubes having closures at their ends which are distal to the external culture medium inlet and outlet means.

Detailed Description of the Invention

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following description of the preferred embodiments taken in connection with the accompanying drawings in which:

FIG. 1 is a side elevation view partly in cross-section showing one embodiment of the static cell culture reactor vessel of the invention.

FIG. 2 is an end view taken along the line 2-2 of FIG. 1.

FIG. 3 is a side elevation view partly in cross-section showing another embodiment of the static cell culture reactor vessel of the invention.

FIG. 4 is an end view of the embodiment shown in FIG. 3.

FIG. 5 is an enlarged side elevation view partly in cross-section showing a portion of the culture reactor vessel of FIG. 3 with a pair of inlet and outlet porous tubes in greater detail.

Now with particular reference to the embodiment shown in FIGS. 1 and 2, reference numeral 10 refers generally to a cell culture reactor vessel which can be used for the static maintenance of mammalian and other animal cells. The reactor shell preferably is made of clear glass or non-toxic rigid plastic materials but also can be made of biocompatible metals such as,

for example, stainless steel. The reactor is shown to have a generally cylindrical shell with sidewall 11 and openings at opposite ends 12 and 13. The cylindrical sidewall can be provided with

5 flared edges 14 and 15 to accomodate wires (not shown) or other such means for reactor support or closure retention purposes. The ends of the cylindrical vessel are shown to be closed with elastomeric stoppers 16 and 17. Stopper 16 is

10 provided with four holes for placement of rigid feed and exhaust tubes into the reactor at one end 12 to serve as inlet and outlet ports for fluid medium and gases. Arrows indicate the preferred direction of flow through these inlet and outlet

15 ports during operation of the reactor. The other reactor end 13 can be permanently sealed but is shown in FIG. 1 to be provided with a stoppered opening as a matter of convenience for additional access to the reactor chamber 18 during downtime

20 of the apparatus. If end 13 is permanently sealed, an alternate opening (other than at end 12) would be preferred for initial packing of the matrix and cells into the reactor chamber. During operation of the reactor vessel, end 13 should be

25 closed.

The reactor 10 is structured to accomodate within its interior chamber 18 a semi-rigid matrix having interstices for retaining cells and for passage of fluid nutrient medium. In order to

30 supply nutrient medium to the cells, a first porous tube 19 which is open at its proximal end and in fluid communication with external inlet port 21 is centrally disposed in the matrix zone

substantially throughout the entire length of said
zone. Porous tube 19 is closed at its distal end
20 but this closure can have the same porosity as
the sidewall of tube 19. The pore size of tube 19
should be such as to permit perfusion of nutrient
fluid through its porous walls into the matrix. A
porous porcelain tube, a porous cellulose,
polytetrafluoroethylene, or polysulfone hollow
capillary membrane tube, or other such porous tube
having a relatively small pore size of from about
0.2μ to about 5μ is preferred to establish a
pressure drop across the tube wall to enhance
uniformity of medium distribution. Smaller pore
sizes, even as low as about 0.01μ, can be used but
are less preferred due to the tendency to clog if
particulate matter in excess of such pore size is
present in the fresh nutrient medium.

A second porous tube 22 which is concentric
to and envelopes porous tube 19 also is disposed
substantially throughout the entire length of the
matrix zone. Tube 22 can be closed at its distal
end but preferably is open for convenient access
to the matrix zone through reactor end 13 during
downtime of the apparatus as stated hereinbefore.
The outer diameter of tube 22 is slightly less

than the inner diameter of the reactor shell to
provide an annular channel 23 for passage of
expended (spent) media and cell product through
external outlet port 24. The pore size of tube 22

5 should be larger than the pore size of tube 19
and, preferably, also has a larger surface area to
facilitate removal of spent media and biomolecular
cell products from the reactor vessel. A porous
porcelain tube or other such porous tube having a

10 relatively large pore size of from about 10µ to
about 150µ is preferred. This larger pore size
and surface area minimizes the pressure drop
across the exit tube. The semi-rigid structure of
the matrix and maintenance of a relatively low

15 flow rate in the nutrient medium tends to prevent
cells from migrating through the high porosity
tube 22.

It will be appreciated that the relative
positions of the first and second porous tubes can

20 be reversed such that the second porous tube 22 is
the low porosity tube and the first porous tube 19
is the high porosity tube. In such configuration,
port 24 will be a fluid medium inlet port and port
21 will be a fluid medium outlet port.

25 It is preferred that the maintenance reactor
10 be operated in a generally horizontal position
with the effluent outlet port 24 being positioned

near the top of the reactor. In FIG. 1, the
reactor is shown in a generally vertical
orientation when the drawing sheet is held in an
upright position for the clearest illustration of
5   the invention due to the extended length of the
reactor relative to its diameter.

In order to supply oxygenated gaseous medium
to the cells, a selectively-permeable tubular
membrane 25 also is disposed substantially
10   throughout the entire length of the matrix zone.
Membrane 25 should be gas-permeable, substantially
liquid impermeable; such properties can be
provided by silicone rubber tubing, e.g.,
Dow-Corning Silastic ® medical grade tubing.
15   Silicone rubber tubing of about one mm inside
diameter and about 2 mm outside diameter is
preferably used. In the embodiment shown in FIGS.
1 and 2, a single length of tubular membrane 25 is
coiled around porous tube 19. Tubular membrane 25
20   is shown to be attached at its ends to rigid tubes
26 and 27 which constitute, respectively, the gas
inlet and outlet ports through stopper 16.

To provide desirable spacing and support for
the porous tubes 19 and 22 and the tubular
25   membrane 25 within the reactor interior, a
silicone rubber septum 28 is positioned close to
the reactor end 12 and adjacent to the inner side

of stopper 16. Septum 28 is provided with four
holes which coincide with the four holes in
stopper 16. A notch 29 provided in the outer
periphery of the septum facilitates collection of
5 effluent from the annular channel 23 through
external outlet port 24. The peripherally flanged
portion of the septum is adapted to receive the
annular proximal end 30 of porous tube 22. The
septum also provides a fluid sealing relationship
10 between the external effluent and internal matrix
zones.

Additional support for porous tube 19 is
provided by a stainless steel tube 31 which is
inserted through external inlet port 21 of stopper
15 16 and disposed lengthwise into tube 19. The
length of tube 31 extending into the reactor
interior should be shorter than tube 19 to permit
the incoming nutrient medium to flow around the
distal end of tube 31 and reach the entire inner
20 periphery of the concentric porous tube 19.
Spacing for tube 19 can be maintained by sealing
it to tube 31 at proximal end 32. Positioning of
an elastomeric O-ring seal 33 between the distal
end 34 of tube 22 and elastomeric stopper 17
25 provides a fluid sealing relationship between
interior chamber 18, annular channel 23 and
stopper 17. Wires (not shown) or other such
retaining means can be placed around flared edge
15 and stopper 17 to further facilitate such fluid
30 sealing relationship.

The semi-rigid matrix can consist of any
finely divided, non-toxic solid material which can
be packed into the reactor chamber to retain the
animal cells in a relatively static or fixed

position within the interior of the reactor vessel
and which provides interstices to permit passage
of fluid nutrient medium through the matrix.
Glass or silica beads, polymeric gel filtration

5   beads such as Sephadex ® cross-linked dextran and
Bio-Gel ® polyacrylamide beads, and cell culture
microcarrier beads such as DEAE-Sephadex,
Cytodex ® charged dextran, and Bio-Carrier ®
acrylamide beads are examples of suitable matrix

10   materials which are commercially available. The
U.S. Patents cited under the Background of the
Invention, above, further describe other such well
known microcarriers which are suitable for use in
the present invention. The preferred

15   microcarriers are polymeric, generally spherical
particles having a diameter of from about 30μ to
about 400μ.

In the method of the present invention
illustrated by the embodiment shown in FIGS. 1 and

20   2, nutrient medium is supplied through external
inlet port 21 into the lumen of porous tube 19.
The nutrient medium can be conveniently pumped
from a fresh medium reservoir (not shown) as
desired. The fresh medium perfuses through the

25   porous walls of relatively low porosity tube 19
into the semi-rigid matrix which contains the
desired animal cells interspersed between
microcarriers or other such finely divided

materials which constitute the matrix.

By such means, the entire nutrient medium makes
cell contact prior to exiting the reactor chamber
through porous tube 22 and outlet port 24. This

5   is distinguished from conventional flow-through
hollow fiber membrane devices wherein only a
portion of the nutrient medium diffuses through
the membrane wall to reach the cells and the
remainder passes through the continuous hollow

10   fiber lumen to the outlet port at the other end of
the device.

The cells and microcarriers can be
initially charged into the interior chamber 18 of
the reactor vessel through end 13 by removal of

15   stopper 17. Prior to introduction into the cell
culture maintenance reactor of this invention, the
cells can be propogated by conventional means such
as, for example, suspension culture or
microcarrier attached suspension culture in

20   separate cell proliferation systems. The
individual cells or the cells attached to the
microcarriers are concentrated, mixed with matrix
material and then introduced into the static
maintenance reactor vessel of this invention.

25   Oxygenation or aeration of the cells is
achieved by circulating oxygen, air or other such
oxygenated gaseous medium through
selectively-permeable membrane 25. Spent medium
and cell biomolecular product then exits the

reactor vessel by perfusion through the porous
wall of the relatively high porosity tube 22,
passage into annular channel 23 and withdrawal
through external outlet port 24. The spent medium
5   can be conveniently collected in an effluent
reservoir (not shown), and cell product can then
be isolated and purified from the spent medium as
desired by conventional means such as, for
10   example, adsorption, extraction, ion-exchange
chromatography, immunoaffinity chromatography, gel
filtration and electrophoresis.

FIGS. 3-5 show another embodiment of the
static cell culture maintenance reactor vessel.
15   As distinguished from the embodiment of FIGS. 1
and 2, the reactor vessel in FIGS. 3-5 contains a
plurality of both the first porous tubes and the
second porous tubes. This embodiment thus is
generally adapted to a larger scale operation than
20   the former embodiment.

In FIGS. 3-5, reference numeral 35 refers
generally to a static cell culture maintenance
reactor vessel having a generally cylindrical
shell with sidewall 36, an endwall 37 and a
25   removable disc-shaped endplate 38. The reactor
vessel 35 accomodates in its interior chamber 39 a
semi-rigid matrix having interstices for holding
cells and for passage of fluid nutrient medium. In

order to supply fresh nutrient medium to the
cells, a group of twelve relatively low porosity
tubes 40 is provided. A group of nine relatively
high porosity tubes 41 is provided for removal of
5 spent medium and cell product. These tubes are
closed at their distal ends (distal to endplate
38). They are arranged substantially in parallel
relationship to each other in the matrix zone and
preferably are spaced apart such that a spent
10 medium tube is positioned within about 2 cm
lateral distance from each fresh medium tube. A
sufficient number of fresh medium tubes preferably
are provided in the reactor vessel such that the
major portion of the matrix volume will be within
15 about 2 cm lateral distance from a fresh medium
tube. A shorter lateral distance (less than 2 cm)
between the fresh medium and spent medium tubes is
preferred when hollow capillary membranes are
employed as the porous tubes such as, for example,
20 hollow fiber membranes having a diameter of from
about 100 to 1000 microns. The porous tubes
should extend substantially throughout the length
of the matrix (or width in a vessel having a
chamber diameter greater than its length).

To provide oxygenation or aeration of the
cells, a length of selectively-permeable membrane
tubing 42 is intricately wound around the porous
fresh medium and spent medium tubes. Inlet and
outlet ports for this tubing are shown,
respectively, at 43 and 44. For clarity, only a

few windings of the membrane are shown. In
practice, more than two hundred linear feet of
silicone rubber tubing per foot of reactor vessel
length have been used. Oxygen or air is flowed

5   through the silicone rubber tubing under pressure
of from about 0 to about 20 lb/in^2 gauge (0 to
about 1.5 K/cm^2) and allowed to diffuse through
the walls of the tubing into the surrounding
matrix zone. Preferably, no point in the matrix

10   zone exists at a distance greater than about 2 cm,
and more preferably less than about 1 cm, from the
oxygen supply tube.

Introduction of the cells and matrix material
into the interior of reactor vessel 35 can be had

15   through a matrix inlet port 45 located near the
bottom of the vessel. A matrix overflow port 46
is positioned near the top of the vessel. This
port also can be used for introduction of
additional matrix material after settling of

20   material initially introduced through inlet
port 45.

The removable endplate 38 of the reactor
vessel is conveniently attached to the reactor
vessel by a split-ring bracket 47 which encircles

25   the reactor vessel at its flanged end 48 and a set
of fasteners 49 equidistantly spaced apart
circumferentially through holes 50 in the bracket
and holes 51 in the periphery of the endplate
(e.g., nut and bolt assemblies as illustrated).

-17-

An annular fibre cushion 52 is shown to be
positioned intermediate the bracket and the
reactor vessel to prevent breakage of a glass
reactor wall from the sealing pressure of a metal

5   bracket in the illustrative embodiment. An
elastomeric O-ring seal 53 positioned between the
lip of the flanged reactor end 48 and an
indentation of the endplate 38 provides a fluid
sealing relationship between the endplate and the

10   reactor interior upon closure with fasteners 49.
An optional port 54 can be positioned in the
sidewalls 36 for removal of air bubbles.

FIG. 5 shows one pair of the porous tubes
40 and 41 in greater detail. Arrows indicate the

15   preferred direction of fluid flow of nutrient
medium through these tubes and the matrix zone
during operation of the reactor. In this enlarged
view, the porous tubes 40 and 41 are shown to be
supported with rigid tubes 55 and 56,

20   respectively, which can be fabricated of stainless
steel or other such rigid material. These rigid
tubes are inserted through openings in the
endplate 38 and disposed lengthwise into the

porous tubes. The length of tubes 55 and 56
extending into the reactor interior is shorter
than tubes 40 and 41 to permit the fresh nutrient
and expended medium to flow around the distal end
5   of the rigid tubes. Alignment of the high
porosity tube 41 with rigid tube 56 is facilitated
by silicone rubber spacers 57 which are notched to
permit passage of medium. Silicone rubber seals
58 and 59 are used to seal the porous tubes to
10   rigid tubes 55 and 56. Silicone rubber seals 60
and 61 also are provided at the distal ends of
tubes 40 and 41, respectively, to provide closures
of these tubes. Alternatively, these closures can
have the same porosity as tubes 40 and 41.
15   Swagelok ® unions 62 and 63 are shown to be welded
into the endplate 38 to seal the rigid tubes 55
and 56, respectively, and to facilitate convenient
assembly of the reactor.

In order to illustrate the operation of the
20   reactor vessel of FIGS. 3-5, cells are first grown
in a separate vessel or cell culture system, e.g.,
the cell culture vessels and systems shown in U.S.
Patents 4,289,854 and 4,335,215. Cells can be attached
to microcarriers (e.g., human diploid fibroblast
25   cells such as FS-4 or AG1523 cells) or free (e.g., SK-
HEP-1 human liver cells or hybridoma cells). These

cells are then mixed at high concentration (e.g.,
10 to 200 ml packed cells per liter) with a matrix
material (e.g., Sephadex G-10, G-25 or G-50
beads). The cell-matrix slurry is then pumped
5   into the reactor vessel 35 through inlet port 45
to completely fill the interior chamber 39 of the
reactor. Excess fluid can be allowed to empty
through the low porosity perfusion tube 40 and
discarded. If sufficient cell-matrix slurry to
10   fill the reactor interior is not available,
additional matrix slurry can be pumped into the
vessel through upper port 46. After filling and
settling of matrix, additional matrix is again
pumped in through port 46 to completely fill the
15   reactor.

Before normal operation of the maintenance
reactor is instituted, the inlet port 45
preferably is disconnected from the cell-matrix
supply source and a reverse flow of fresh medium
20   from the high porosity tubes 41 to the low
porosity tubes 40 is instituted to remove
unattached cells from the immediate vicinity of
the high porosity tubes. During operation of the
maintenance reactor vessel, fresh medium is pumped

directly or through a manifold from a fresh medium
reservoir (not shown) into the low porosity tubes
40. The pressure drop across the low porosity
tubes caused by this fresh medium flow maintains
5   uniformity of perfusion throughout the reactor.
Effluent product flows from the cell-matrix into
the high porosity tubes 41 and out into a
product/effluent reservoir (not shown). The
pumping of medium during operation of the reactor
10 can be carried out continuously or periodically at
a flow rate sufficient to provide the nutrients
required for survival of the particular cells
being maintained in the reactor and to remove the
cell product.

15   Cells can be maintained in this system for
long periods of time with product continually
harvested from the spent medium. In a 15.24 cm
long, 15.24 cm diameter cylindrical reactor vessel,
cells from 100 to 400 liters of conventional
20 suspension culture can be maintained. In a
81.28 cm long, 15.24 cm diameter cylindrical
reactor vessel, cells from 500 to 2000 liters of
conventional suspension culture can be maintained.

While horizontal alignment of the reactor
vessel allows extension of the length without
changing perfusion parameters, other geometries of
the system can be utilized. For example, the
5   cylinder can be very large in diameter and
relatively short in length (or width) but stand in
a vertical position. It is preferable to minimize
the hydrostatic pressure effects in the perfusion
tubes so as to prevent non-uniformity of medium
10   perfusion.

The apparatus of this invention can also
employ auxiliary features such as, for example, pH
and dissolved oxygen sensing electrodes, sampling
ports, in-line air filters and the like cell
15   culture system features.

The following detailed examples will further
illustrate the use of the above-described static
cell culture maintenance system although it will
be understood that the invention is not limited to
20   these specific examples.

-22-

EXAMPLE 1

A micro-static maintenance reactor (M-SMR)
was constructed as shown in FIGS. 1 and 2. The
reactor consisted of a Pyrex ® outer cylindrical

5   shell about 165.1 mm long and 25.4 mm outside
diameter and contained single high and low
porosity tubes. The high porosity tube was about
127 mm long with 17.5 mm outside diameter and
3.18 mm wall thickness. This tube had a nominal

10   pore size of 20 microns (Kynar porous plastic
tubing from Portex Technologies, Division of
Glass Rock, Medical Services, Fairburn, GA 30213).
Contained within the high porosity tube was a
low porosity tube with 0.555 cm OD and 0.0794 cm

15   wall thickness. This microporous porcelain tube
had a porosity of nominally 0.8 microns and was
purchased under catalog #105779-04 from Selas,
Flotronics Division, Huntingdon Valley, PA 19006.
Approximately 60.96 cm of medical-grade silicone

20   rubber tubing 1 mm ID and 2 mm OD were wrapped
around the inner porous tube. This tubing was pur-
chased under catalog #518-145 from Patter Products,
Inc., Beaverton, MI 48612. The working volume
of this reactor between the inner and outer

25   porous tubes was approximately 10 ml.

The cells used in this run were an
anchorage-independent hybridoma cell line
designated 1-15 2F9 and developed by R. Kimes and
J. Olander of Monsanto Company. This mouse-mouse

hybridoma produces an IgG monoclonal antibody against an antigen associated with a human hepatoma cell line (SK-HEP-1). Background information on the illustrative SK-HEP-1 cell

5 line can be had by reference to U.S. Patent 4,209,587. The hybridoma culture was grown to approximately 10^6 cells/ml in a 500 ml conventional spinner and collected by gentle centrifugation at 200 rpm into the matrix material. This matrix

10 material consisted of Sephadex ® G-50 chromatography beads which were sterilized by autoclaving in phosphate buffered saline (PBS) and washed with the nutrient medium used for perfusion in this Example. Dulbecco's modified MEM medium with

15 4.5 grams/liter glucose supplemented with 6% fetal bovine serum was used without antibiotics. The pellet of cells and chromatography beads was retained in the conical portion of a centrifuge bottle after aspiration of the supernatant. A dense slurry of cells and

20 beads was then pipetted into the M-SMR device with removal of some bead-free effluent. This cell containing effluent was used to wash in more of the bead slurry. As the pore size of the high porosity tube was larger than the average size of

25 the hybridoma cells, portions of these cells

migrated through the walls of the high porosity
tube into the effluent during the inoculation
procedures. However, after sealing the reactor
and initiating perfusion of nutrient medium,

5   relatively few hybridoma cells were lost. While
determination of exact values of the cell inoculum
are not possible due to losses in centrifugation
and through the high porosity tube, it is
estimated that between $2x10^8$ and $5x10^8$ cells were

10   retained within the 10 ml operating volume of the
reactor. Viability of these cells by dye
exclusion was estimated to be approximately 80%.
Medium was perfused through the reactor at a rate
of approximately 2 mls/hr and a gas mixture flowed

15   through the silicone rubber tubing at a rate of
1-2 mls/min. This gas mixture consisted of carbon
dioxide, oxygen and air such that average
concentrations of the input gas were 37 ± 12 mm Hg
carbon dioxide and 310 ± 46 mm Hg oxygen. These

20   concentrations were measured in an IL blood-gas
analyzer (Instrumentation Laboratories). During
the two-week run of this reactor, samples were
taken at 1-3 day intervals for measurement of pH

and dissolved gas concentrations and also for determination of antibody levels. During the run, the average pH value was 7.13 ± 0.11, the dissolved CO_2 was 58.7 ± 14 mm Hg and the oxygen level was 116 ± 13 mm Hg. These results indicate that culture parameters were maintained within desired operating ranges during the entire growth period (desired pH between about 6.9 and 7.3; desired oxygen between about 20 mm Hg and 160 mm Hg; and desired CO_2 between about 35 mm Hg and 100 mmHg).

Samples removed from the reactor were also tested for the presence of monoclonal antibodies by two separate ELISA assays (Enzyme Linked Immunoabsorbent Assay). The first assay detected the presence of mouse immunoglobulin and consists of the following: unlabeled goat anti-mouse immunoglobulin is bound to microtiter plates overnight at $4^{\circ}C$. The plates are washed and then blocked within 0.1% solution of bovine serum albumin to reduce non-specific binding. After an additional wash, the sample is added and incubated for 2 hrs. at room temperature. After washing, labeled goat anti-mouse immunoglobulin (labeled with alkaline phosphatase) is added for an additional 2 hr. incubation. The latter material is washed off and p-nitrophenyl phosphate is added as a substrate and incubated for 30 min. at room

temperature. The optical density of the colored
reaction is read with a microtiter optical reader
at 410 nm. The second ELISA assay involves
specific recognition of the human hepatoma antigen

5   against which mice were immunized for preparation
of this hybridoma. In this case, the hepatoma
antigen is bound at 4°C overnight to the
microtiter plates and all successive steps are the
same as above. Substantial amounts of monoclonal

10   antibody were detected by both assays in the
effluent medium flowing from the reactor. Levels
the same or greater than found in conditioned
media from conventional culture of the hybridoma
cells were shown. These results, indicate

15   long-term sustained production of the specific
monoclonal antibody by cells maintained at high
density in the M-SMR system. At the conclusion of
the run, approximately $2x10^8$ viable cells were
recovered.

20   <u>EXAMPLE 2</u>

A large scale static maintenance reactor
(SMR) with a working volume of approximately 2
liters, as shown in FIGS. 3-5, was used to
maintain an anchorage-dependent cell line for a

25   period of about two months. The reactor vessel
shell consisted of a 15.24 cm long, 15.24 cm diameter
cylindrical Pyrex glass pipe end cap (Corning

#72-6300). The high and low porosity tubes were constructed of the same material and had the same diameters as used in Example 1 but consisted of an array of 12 low porosity tubes and 9 high porosity tubes. 33.528 meters to 36.576 meters of silicone rubber tubing as used in Example 1 was wound randomly around these porous tubes. During operation, a pressure drop of approximately $0.15 kg/cm^2$ was used across this silicone rubber tubing for a flow of approximately 10 mls of gas/min.

The AG1523 human foreskin fibroblast cell line used in this Example was obtained from the Institute for Medical Research, Camden, NJ at passage three. These cells were grown in T flasks and roller bottles in accordance with the procedure described in U.S. Patent 4,273,871. Larger numbers of cells were produced in a 4-liter microcarrier reactor system as described in U.S. Patent 4,335,215. After a cell density of $7.0 \pm 0.6 \times 10^6$ cells/ml was obtained, the cells still attached to the microcarriers (polyacrylamide Bio-Carriers ®,

BioRad Laboratories, Richmond, CA) were mixed with additional Bio-Carriers to provide a total settled volume of approximately 1800 mls. This thick slurry was pumped into the static maintenance reactor from the bottom while air was removed from an upper port. Additional matrix material, consisting of Sephadex G-50 chromatography beads, was used to completely fill the reactor. During the filling operation, excess liquid was allowed to flow out of the low porosity tubes into a medium supply manifold and out to a discard vessel. After the total reactor volume was solidly packed with matrix material containing the approximately 2.8×10^{10} cells, a pressure overflow vessel was attached to an upper port and partially filled with Sephadex G-50 matrix slurry. This vessel was sealed with a 0.4kg/cm^2 check valve provided to prevent overpressurization of the reactor. Medium flow was then established through the reactor from the low porosity tubes to the high porosity tubes and out through an exterior manifold containing an INGOLD pH electrode into an effluent holding vessel. Initially, approximately 4 liters fresh medium were pumped over a 2 hr period to saturate the entire matrix and cells with fresh medium. Dulbecco's modified MEM medium with 4.5 grams/liter glucose supplemented with 6%

fetal bovine serum was used without antibiotics. A flow rate for liquid medium between 1.5 and 3 mls/min was used throughout the 62-day run. The average liquid flow rate, as measured every two days during sampling, was 2.18 ± 0.38 mls/min. Both oxygen and carbon dioxide concentrations were measured in the gas before entering the reactor and after traversing the 35.528-36.576 meters of silicone rubber tubing. A gas flow of approximately 10 ml/min was maintained throughout the SMR run with a pressure differential of approximately 0.15 kg/cm^2 across the reactor. Samples of gas were read in an IL blood-gas analyzer and indicated a continual depletion of oxygen and increase of CO_2 throughout the run. Oxygen consumption by the reactor determined both from the change in the 10 ml/min gas flow and in the change in the approximately 2 ml/min medium flow was an average of $2.1 \pm .08 \times 10^{-5}$ moles of oxygen/min. Similar calculations could not be made for the carbon dioxide due to the evolution of CO_2 from the bicarbonate buffer with change in pH across the reactor. However, CO_2 concentration ranged from about 45 mm Hg in the input gas to 60-70 mm Hg after traversing the reactor. Input gas levels were about 40-45 mm of Hg of carbon dioxide and 250-300 mm Hg for oxygen.

After the initial 18 liters of serum-containing
medium perfused through the reactor over
approximately 6-1/2 days, serum-free medium
supplemented with 0.5 mg/ml bovine serum albumin,

5   0.5 µg/ml insulin and 0.5 µg/ml human transferrin
and 5 nl/ml linoleic acid was used. This
serum-free medium was perfused for 24 days; during
the final 32 days the 6% fetal bovine serum
supplemented medium was used. Oxygen consumption

10   and CO_2 production, as well as pH levels, remained
substantially constant throughout the entire
two-month period.

During the two month run, samples were taken
and assayed for angiogenesis factor and

15   plasminogen activator. The assay for the
angiogenesis factor was by stimulation of
endothelial cell growth as described by J.V.
Olander, J.C. Marasa, R.C. Kimes, G.M. Johnston
and J. Feder, "An Assay Measuring the Stimulation

20   of Several Types of Bovine Endothelial Cells by
Growth Factors Derived from Cultured Human Tumor
Cells," In Vitro 18, 99-107 (1982). Substantial
levels of endothelial cell growth stimulatory
activity were observed throughout the run with the

highest value occurring after reintroduction of serum supplemented media. From about a 2-fold to a 5-fold increase in endothelial cell number was obtained in comparison to controlled media (fresh growth media unexposed to the cells) during the maintenance period.

Plasminogen Activator (PA) activity can be determined by the fibrin plate method of Astrup, <u>Arch. Biochem. Biophys.</u> <u>40</u>, 346-351 (1952) and variations thereof as described, for example, in U.S. Patent 3,778,352. In this general method, the plasminogen activator in a sample is determined by measuring the diameter of a clear radial diffusion zone in a gel plate or dish caused by fibrinolysis of a known amount clotted fibrinogen in the gel. Such a clearing zone assay was used to detect plasminogen activator in samples from the above run as follow: Sea plaque agarose was mixed with fibrinogen, thrombin and plasminogen and then poured into a large petri dish and allowed to gel overnight. Reaction between the thrombin and fibrinogen formed a fibrin matrix within the agarose gel. Five μl samples of conditioned media from the static maintenance reactor run were spotted on this gel

along with varying known concentrations of a
urokinase standard solution. The gel was
incubated at 37°C. overnight. Any plasminogen
activator in the samples would catalyze the
5 production of plasmin from the plasminogen
contained within the gel. Plasmin then dissolved
the fibrin matrix forming a circular cleared zone
around the sample spot. After incubation, the gel
was stained with 0.1% amido black at room
10 temperature for 2 hrs in a solution of 70%
ethanol, 10% acetic acid, 20% water and then
destained in the same solvent for one day. The
diameter of cleared circular zones around sample
spots was compared with similar zones around known
15 concentrations of urokinase. Substantial levels
of plasminogen activator activity were found
during the entire 24 days of serum-free perfusion,
but the levels detected during the perfusion
period in the presence of fetal bovine serum
20 generally were lower. Fetal bovine serum contains
an inhibitor to PA activity which accounts for the
reduced response in its presence.

Plasminogen activator activity on samples from the above run was further confirmed by the measurement of the release of ^{125}I-fibrin in the modified fibrin dish assay method as described by
5 Feder et al., <u>Biochem. Biophys. Res. Commun.</u> <u>83</u>(3) 1164-1170 (1978).

Conditioned medium from both fetal bovine serum containing portions of the growth and serum-free portions of the reactor run was
10 concentrated on an Amicon hollow fiber system to retain materials above 10,000 molecular weight. These concentrates can also be assayed for various other activities. Thus, these concentrates were found to have positive activity for vascular
15 permeability factor according to the method described by Dvorak et al., <u>J. Immunol.</u> <u>122</u>(1), 166-173 (1979).

It should be understood that all types of animal cells can be maintained by the method and
20 apparatus of this invention such as, for example, mammalian, amphibian and avian cells. In addition to the cells illustrated in the foregoing Examples, other cells can be employed which are

representative of hybridomas, primary cultures of normal and neoplastic cells, transformed and nontransformed animal cell lines, such as human lung fibroblast (WI-38) cells, rhesus monkey

5   kidney (MK-2) cells, cervical carcinoma (HeLa) cells, baby hampster kidney (BHK-21) cells, simian virus 40 transformed 3T3 mouse embryo fibroblast (SV3T3) cells, chick embryo fibroblast cells, and the like. So also, the apparatus and method of

10   the invention is adapted for use with any conventional cell nutrient medium such as, for example, Eagle's basal medium, Dulbecco's modified Minimum Essential Medium (MEM), Medium 199, RPMI 1640 medium and the like.

15   Still other embodiments and examples of the invention will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention and it will be understood that all such

20   further embodiments and examples are included within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of maintaining animal cells in vitro in a substantially arrested state of proliferation with continuous secretion of cell product which comprises:

5   (a) suspending said cells in a semi-rigid matrix disposed in a cell culture reactor chamber and having interstices for passage of fluid nutrient medium,

10   (b) supplying fresh nutrient medium for said cells by passing said nutrient medium through an external inlet in the reactor into the lumen of at least one first porous tube disposed in said chamber substantially throughout the expanse of said matrix and perfusing said nutrient medium 15 through the walls of said porous tube into said matrix,

    (c) withdrawing expended nutrient medium and cell product from said matrix by perfusing through the walls of at least one second porous 20 tube disposed in said chamber substantially throughout the expanse of said matrix and passing said spent medium and cell product through an external outlet in said reactor, said second porous tube having a pore size larger than the 25 pore size of said first porous tube, and

(d)   supplying oxygenated gaseous medium for said cells by perfusing into said chamber through the walls of at least one selectively-permeable tubular membrane disposed in said matrix substantially throughout the expanse of said matrix and having openings at both ends of its lumen for inlet and outlet of said gas to and from the outside of said reactor.

2.   The method of Claim 1 in which said second porous tube is concentric to said first porous tube.

3.   The method of Claim 1 in which a plurality of first porous tubes and a plurality of second porous tubes are disposed in substantially parallel relationship in said matrix.

4.   The method of Claim 1 in which the pore size of the first porous tube is from 0.2 μ to 5 μ and the pore size of the second porous tube is from 10 μ to 150 μ.

5.   The method of Claim 3 in which the pore size of the first porous tubes is from 0.2 μ to 5 μ and the pore size of the second porous tubes is from 10 μ to 150 μ.

6.   The method of Claim 1 in which the semi-rigid matrix comprises a packed volume of polymeric generally spherical microcarriers having a diameter of from 30 μ to 400 μ.

7. The method of Claim 1 in which the animal cells are mammalian hybridoma cells and the cell product is monoclonal antibody produced by said cells.

8. The method of Claim 1 in which the animal cells are mammalian fibroblast cells and the cell product has angiogenic or plasminogen activator activity.

9. Apparatus for maintaining animal cells in vitro in a substantially arrested state of proliferation with continuous secretion of cell product which comprises a housing, a chamber
5 within said housing for holding cells in a semi-rigid matrix with interstices for passage of fluid culture media, external culture medium inlet and outlet means disposed in a wall in said housing, said inlet means being in fluid
10 communication with at least one relatively low porosity tube disposed substantially throughout the expanse of said chamber, said outlet means being in fluid communication with at least one relatively high porosity tube disposed
15 substantially throughout the expanse of said chamber, external gas inlet and outlet means disposed in a wall in said housing and in gaseous communication with a selectively-permeable membrane disposed substantially throughout the
20 expanse of said chamber, said relatively low and relatively high porosity tubes having closures at their ends which are distal to the external culture medium inlet and outlet means.
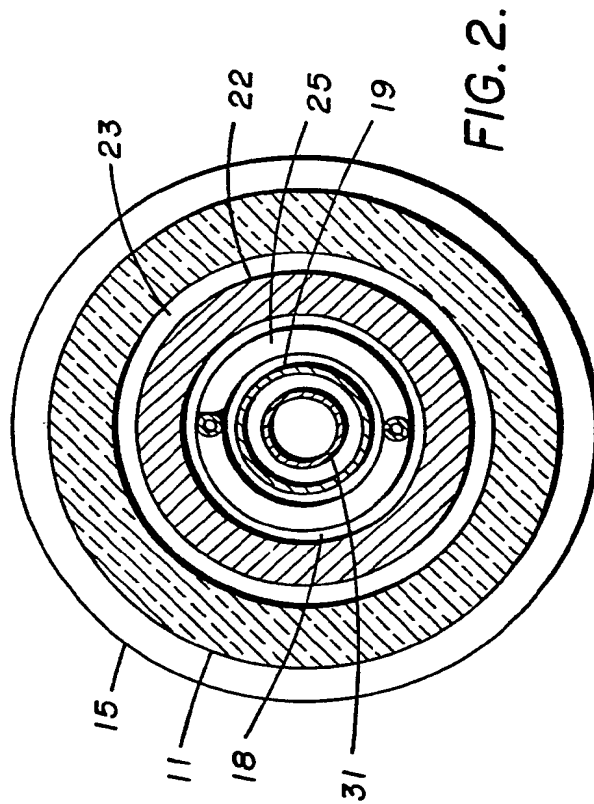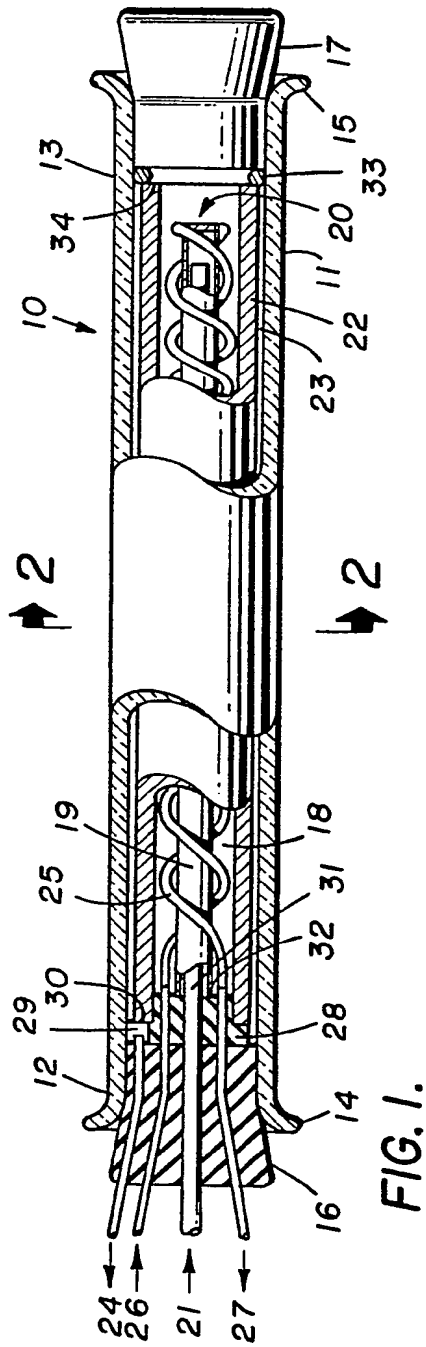
10. The apparatus of Claim 9 in which said relatively high porosity tube is concentric to said relatively low porosity tube.
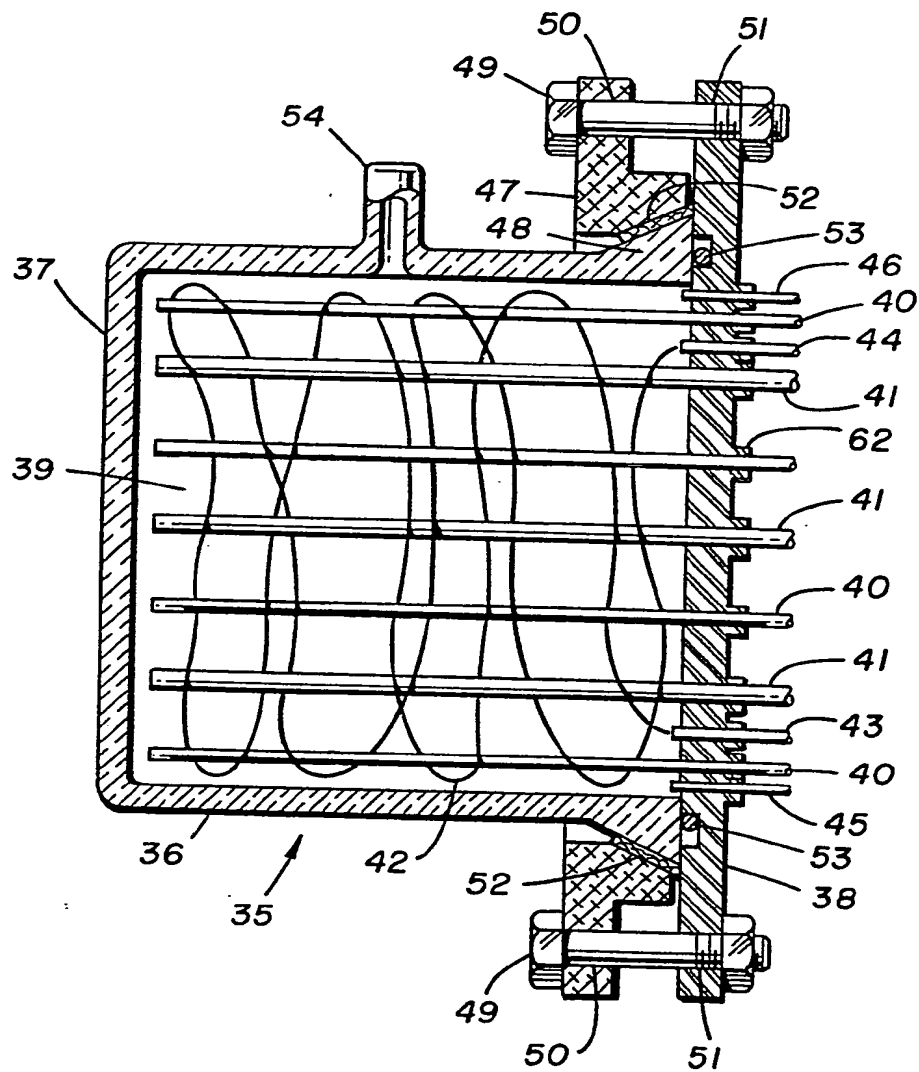
11. The apparatus of Claim 9 in which a plurality of relatively high porosity tubes and a plurality of relatively low porosity tubes are disposed in substantially parallel relationship in said matrix.

12. The apparatus of Claim 9 in which the pore size of the relatively high porosity tube is from 10 μ to 150 μ and the pore size of the relatively low porosity tube is from 0.2 μ to 5 μ.

13. The apparatus of Claim 11 in which the pore size of the relatively high porosity tubes is from 10 μ to 150 μ and the pore size of the relatively low porosity tubes is from 0.2 μ to 5 μ.
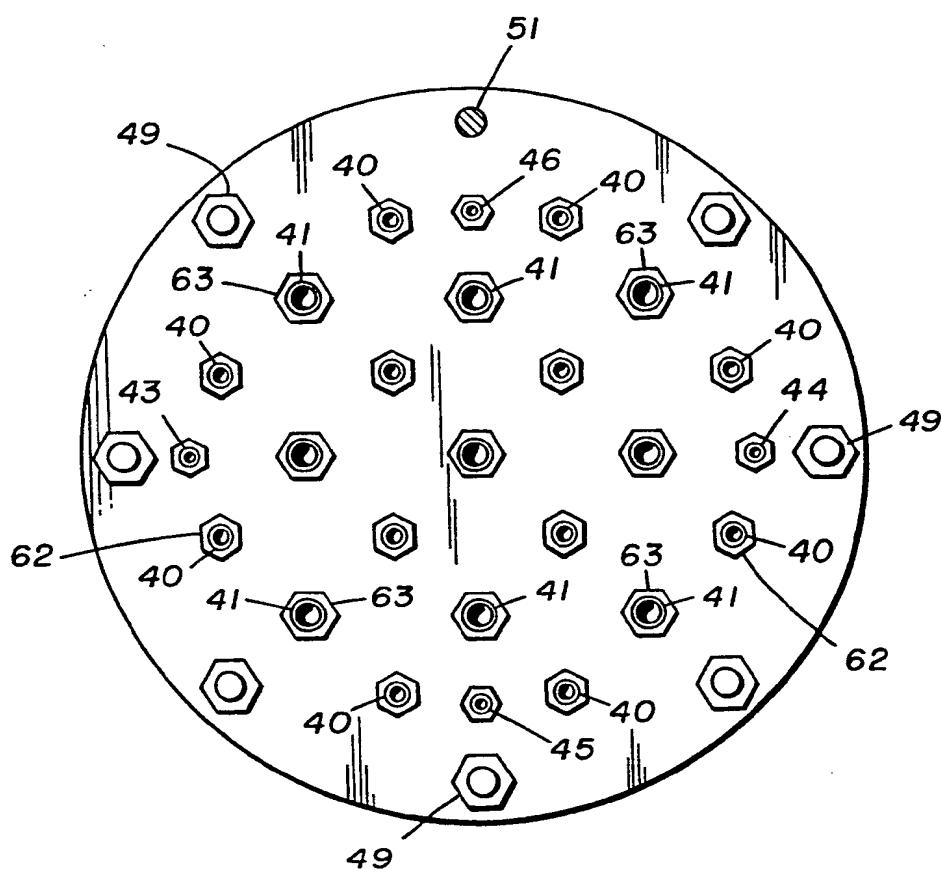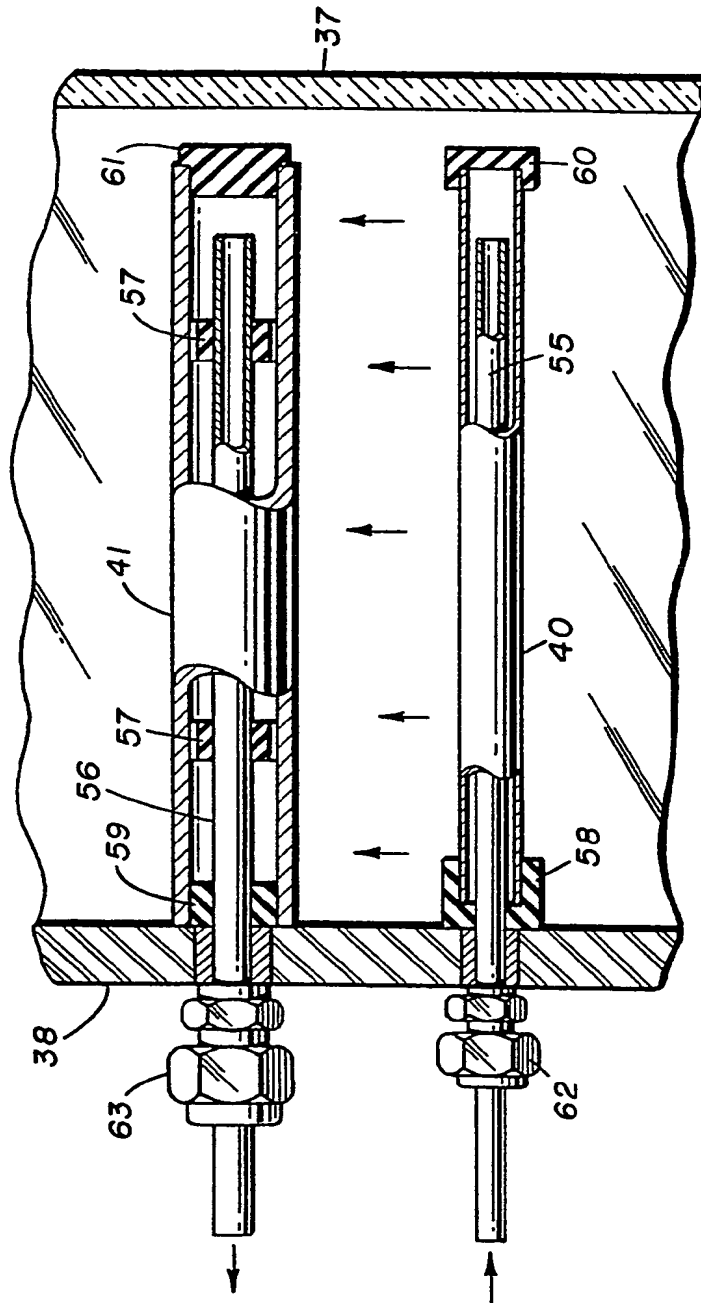
FIG. 1.

FIG. 2.

0113328

FIG. 3.

FIG. 4.

FIG. 5.

THIS PAGE BLANK (USPTO)